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Tumorigenesis and Neoplastic Progression

Stromal Regulation of Neoplastic Development

Age-Dependent Normalization of Neoplastic Mammary Cells by Mammary Stroma

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There is mounting evidence that the stroma plays a crucial role in mammary gland carcinogenesis. Here, we report that mammary gland stroma from mature and multiparous rats prevents neoplastic development and encourages normal ductal growth of grafted epithelial cancer cells. Fifty thousand epithelial cancer cells were injected into the cleared fat pads of virgin hosts at 24, 52, 80, and 150 days of age and of hosts that had undergone two cycles of pregnancy, lactation, and involution. Six months after inoculation, tumor incidence was 75%, 100%, 50%, and 18.2% in 24-, 52-, 80-, and 150-day-old virgin rats, respectively, and 0% in the twice-parous animals. Most remarkably, these neoplastic cells appeared to form normal ducts in all hosts—Ha-*ras*-1 mutation served as a marker to identify the tumor origin of the outgrowths. The tumor development pattern suggests a parallel to the phenomenon of age- and reproductive state-dependent susceptibility and resistance to chemical carcinogens. As susceptibility to carcinogenesis decreases, the ability of the stroma to reprogram neoplastic epithelial cells increases. Thus, the neoplastic phenotype is context-dependent, and it therefore offers the intriguing possibility that the process of carcinogenesis is amenable to normalization or cure once the mechanisms of stroma-mediated normalization are elucidated and manipulated. *(Am J Pathol 2005, 167:1405–1410)*

During early development, the mesenchyme plays inductive and permissive roles in epithelial morphogenesis, differentiation, and proliferation. These events have been observed in experimental models both *in vitro*[1,2] and *in vivo*.[3] During adult life, the stroma plays a comparable role in the maintenance of the structure and function of epithelia.[4,5] An equally prominent role for the stroma has been verified experimentally during the process of carcinogenesis in several organs.[6–9]

Using a tissue recombination model, we and others recently observed that the stroma plays a crucial role in mammary gland carcinogenesis. Specifically, rat mammary adenocarcinomas occurred only when the mammary stroma was exposed to the chemical carcinogen *N*-nitrosomethylurea, regardless of whether the epithelial cells were exposed as well.[9] On the other hand, it has also been shown that carcinoma-associated stromal cells have the capacity to transform nontumorigenic epithelial cells into neoplasms.[10–12] Altogether, these experimental observations support the concept that carcinogenesis and neoplasia are emergent, supracellular phenomena.[13–15]

In a different but related context, the results obtained by Rivera and co-workers[16,17] in the 1980s suggest another role for the stroma, namely, that of normalizing or reprogramming mammary cancer cells *in vivo*. Neoplastic epithelial cells and tissue fragments obtained from primary mammary tumors developed into secondary tumors upon inoculation into cleared mammary fat pads (CFPs).[16,17] Insightfully, Rivera and co-workers[16,17] observed that phenotypically normal ducts were also present in the hosts' CFPs in the recombinant tissues. However, this phenomenon was not investigated further, probably because it could not be explained within the context of the prevailing somatic mutation theory. The main assumption of the somatic mutation theory is that neoplasms are the result of accumulated mutations in the DNA of an epithelial cell. After 2 decades of research highlighting the importance of the extracellular matrix and of stromal-epithelial interactions on the expression and

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suppression of neoplastic phenotypes,[18] Rivera and colleagues'[16,17] observations can now be reinterpreted in the context of the tissue organization field theory, which posits that carcinogenesis is a tissue-based process, akin to development gone awry.[13]

One of the predictions of the tissue organization field theory is that carcinogenesis can potentially be reversed. This would occur when the normal tissue morphogenetic unit (stroma and epithelium) is re-established and the constitutive proliferative ability of epithelial cells is inhibited.[14,19,20] Experimentally, the reversal of neoplastic behavior has been accomplished repeatedly when neoplastic cells were placed within the normal tissues from which they originated. For instance, in a series of elegant experiments, Illmensee and Mintz[21] showed that teratocarcinoma cells injected into blastocysts became integrated into the normal tissues of the mosaic mice. More recently, McCullough and colleagues[22] observed that hepatocellular carcinoma cells formed aggressive tumors when injected subcutaneously but became integrated into the normal tissue when placed into the liver of syngeneic animals. On the other hand, Weaver and collaborators[23] have shown reversion of the malignant phenotype of breast cells *in vitro* by modifying the cell surface β1 and β4 integrins in a three-dimensional basement membrane assay. Spontaneous regression has been reported in almost all types of human neoplasias.[24] Although only a few cases of spontaneous regression of breast cancer have been documented, rigorously conducted recent mammographic studies suggest that this phenomenon may be more common than previously thought.[25–27]

Based on the above background information, we decided to further explore this subject using the rat mammary gland as an experimental model. Thus, we chose to test whether age and parity affects the ability of the stroma to support or repress tumor development. To test their potential to form either normal ducts or neoplasms, we transplanted neoplastic epithelial cells into CFPs of virgin rats of different ages and into animals that had completed two pregnancies (including lactation and involution). This report is part of an extended, detailed effort to map out the influences of the rat mammary stroma on carcinogenesis and tumor regression.

Materials and Methods

Animals

Wistar-Furth (WF) rats were purchased from Harlan (Indianapolis, IN) and housed in transparent plastic cages with food and water *ad libitum*. Animals were maintained on a 14:10 hour light:dark cycle and care was in accordance with the Guidelines for the Care and Use of Animals and the Tufts-New England Medical Center Institutional Animal Care and Use Committee.

Induction of Mammary Tumors

Virgin 52-day-old female rats were injected intraperitoneally with a single dose of 50 mg of *N*-nitrosomethylurea/kg (Sigma, St. Louis, MO) body weight. Tumors were palpable beginning at 12 weeks after treatment. These tumors were designated donor tumors to distinguish them from those tumors derived from the inoculated neoplastic epithelial cells, which were arbitrarily called secondary tumors.

Preparation of Cells for Transplantation

Cells were prepared according to the method described by Alston-Mills and Rivera[28] with minor modifications.[9] Briefly, when tumors reached ~1.5 cm in diameter they were removed and placed in sterile phenol red-free Dulbecco's modified Eagle medium (Irvine Scientific, Santa Ana, CA). The tumors were minced and digested in phenol red-free Dulbecco's modified Eagle medium containing 0.1% collagenase type 3 (Worthington, Lakewood, NJ) at 37°C for 2 hours while agitating. This digest was centrifuged and the pellet was then treated with 1.25% pronase (Calbiochem, San Diego, CA) for 5 minutes at 37°C with agitation. This cell suspension was filtered through a 530-μm pore Nitex filter (Sefar America, Kansas City, MO) and the filtrate was centrifuged at 100 $\times$ *g* for 3 minutes. Subsequent filtrations were performed using a 250-μm pore filter, then a 10-μm pore filter. The cells were counted with a Coulter Counter ZM (Beckman Coulter, Fullerton, CA) and resuspended in Dulbecco's modified Eagle medium.

Hosts for Tumor Cell Transplantation

The mammary epithelium was surgically removed (CFP) from the fourth and fifth right abdominal-inguinal mammary glands of 10-day-old rats, according to procedures that were originally outlined by DeOme and colleagues[29] and done routinely in our laboratory. The left abdominal-inguinal mammary glands were left intact and considered internal controls. In each of the animals used in these experiments, the excised epithelium was whole-mounted and observed microscopically to assure that the ductal tree was removed in its entirety and that only a small portion of the fat pad remained attached to it. The host rats were separated into two groups: one of virgin females of 24, 52, 80, and 150 days of age, and another of twice-parous females (Figure 1). The twice-parous rats were bred starting at 2 months of age. In all these rats, the fourth CFP was used as the transplantation site.

Cell Transplantation

Using a Hamilton syringe (Hamilton Co., Reno, NV), 5×10^4 cells contained in a 10-μl volume were injected into the right side CFP. Starting 1 month after the cell inoculation, all rats that received a cell transplant were palpated weekly. Animals were sacrificed when tumors reached 1 cm in diameter or 6 months after cell transplant, whichever occurred first. Animals were excluded from the analyses when no ductal epithelial outgrowths were found in the whole mounts (no takes) or when they died as a result of surgical complications. The initial (i)

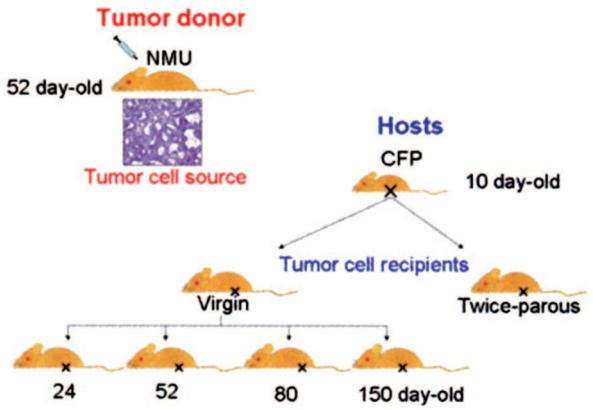

Figure 1. Schematic representation of the experimental design.

and final (f) sample sizes at 6 months after the cell injection were as follows: age: 24 days old, i = 9 and f = 8; age 52 days old, i = 9 and f = 7; age 80 days old, i = 11 and f = 10; age 150 days old, i = 11 and f = 11; twice-parous rats, i = 7 and f = 5.

Whole Mounts and Histology

Whole mounts were prepared following protocols described on the Biology of the Mammary Gland website (*http://mammary.nih.gov*) and by Thompson and colleagues.[30] The mammary glands were removed and spread on a 75 × 50 × 1-mm glass slide (Fisher Scientific, Pittsburgh, PA), fixed overnight in 10% phosphate-buffered formalin, dehydrated in 70%, 95%, and 100% alcohols, cleared in toluene, rehydrated, and stained with carmine alum. After staining, the whole mounts were dehydrated as described above, cleared in xylene, and bagged in Kpak SealPak heat-seal pouches (Kpak Corp., Minneapolis, MN) in methyl salicylate. The whole mounts were analyzed under a stereomicroscope Stemi 2000 (Carl Zeiss, Munchen-Hallbergmoos, Germany). Microscopic lesions found during this analysis were removed and embedded in paraffin for histology. Tumors larger than 0.5 cm were removed before the whole mounts were prepared, separately fixed as described above, and paraffin-embedded. Images were captured with an AxioCam HR color digital camera (Carl Zeis) attached to a stereomicroscope.

*DNA Extraction and Analysis of Ha-*ras*-1 Gene Mutation*

DNA was extracted from the donor tumors, the secondary neoplasms (both palpable tumors and microscopic lesions), and the normal outgrowths using a DNeasy kit (Qiagen Inc., Valencia, CA), following the manufacturer's instructions. We used the mismatch amplification mutation assay described by Cha and colleagues[31] with some modifications. The mismatch amplification mutation assay is specific for the codon 12 GGA to GAA mutation in the Ha-*ras*-1 gene. Briefly, this method uses two sets of primers: one targets the mutation and the other a control area in the genomic DNA. The mutant-specific mismatch primer PAA (5′-CTTGTGGTGGTGGGCGCTGAA-3′), the Pmnl2 (5′-ACTCGTCCACAAAATGGTTC-3′), and the control primers [P1: 5′-CCTGGTTTGGCAACCCCTGT-3′ and Pmnl2: 5′-ACTCGTCCACAAAATGGTTC-3′] were used at a 40 ng/μl concentration. The polymerase chain reaction was performed using Platinum Supermix (Invitrogen, Carlsbad, CA). The polymerase chain reaction products were run in a 2% agarose gel (Life Technologies, Inc., Grand Island, NY). The expected size of the nonmutated Ha-*ras*-1 gene is 128 bp whereas the mutated Ha-*ras*-1 gene is 74 bp.

Table 1. Outcome of Neoplastic Epithelial Cell Injection into Hosts at Different Ages and Parity Status

Host age	Initial no.	Final no.	Tumors	Outgrowths
Twice parous	7	5	0/5 (0%)	5/5 (100%)
150 days old	11	11	2/11 (18.2%)	11/11 (100%)
80 days old	11	10	5/10 (50%)	7/10 (70%)
52 days old	10	8	8/8 (100%)*	7/8 (87.5%)
24 days old	9	8	6/8 (75%)†	5/8 (62.5%)

*Statistically different from twice-parous and 150-day-old host groups.
†Statistically different from twice-parous and 150- and 80-day-old host groups.

Statistics

Statistical significance of the incidence of neoplastic lesions was determined using the χ^2 test in the SPSS software package (Chicago, IL).

Results

Normal Ducts Developed from Tumor Cells

The transplantation of mammary tumor cells into CFPs gave rise to ductal outgrowths that were phenotypically normal at the time of harvesting (6 months after injection of tumor cells). Normal ductal development was observed in almost all animals, regardless of the host's age at transplant or parity status. Ductal outgrowths were not observed in the mammary glands of animals that developed large tumors because the tumors encompassed the entire fat pad at the time of tissue collection. From these data, we cannot rule out the possibility that ductal growth occurred.

Secondary Tumor Development Inversely Correlated with the Age of the Host

The transplanted donor tumor cells gave rise to a variety of outgrowths, ranging from large secondary tumors to microscopic neoplastic lesions as well as normal ductal development. The tumor incidence correlated inversely with the age of the stroma. That is, the highest tumor incidence was observed in the younger animals: 75% of the 24-day-old hosts and 100% of the 52-day-old hosts

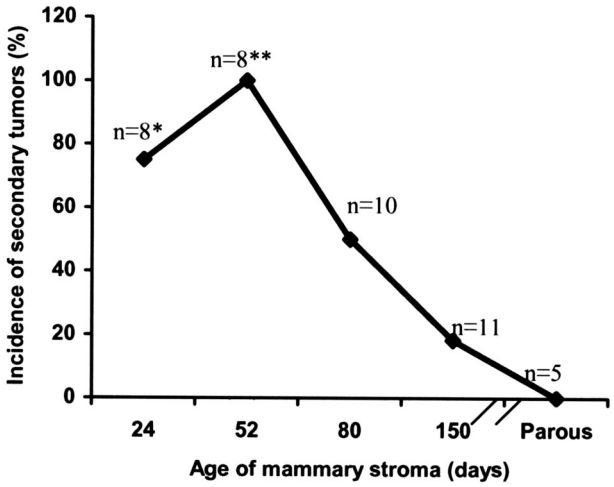

Figure 2. The incidence of secondary tumors decreases with the age of the stroma. The parous host only developed normal ductal outgrowths. *Statistically different from twice-parous and 150- and 80-day-old host groups. **Statistically different from twice-parous and 150-day-old host groups.

developed secondary tumors (Table 1, Figure 2). The incidence of secondary tumors decreased to 50% in the 80-day-old hosts and to 18.2% in the 150-day-old hosts. The twice-parous group only developed phenotypically normal ducts; no tumors or microscopic neoplastic lesions were observed in this group. Statistically significant differences were observed between the 52-day-old group and the parous ($P = 0.001$), the 150-day-old ($P = 0.001$), and the 80-day-old ($P = 0.029$) groups. The 24-day-old group was different from the parous ($P = 0.016$) and the 150-day-old ($P = 0.022$) groups (Table 1).

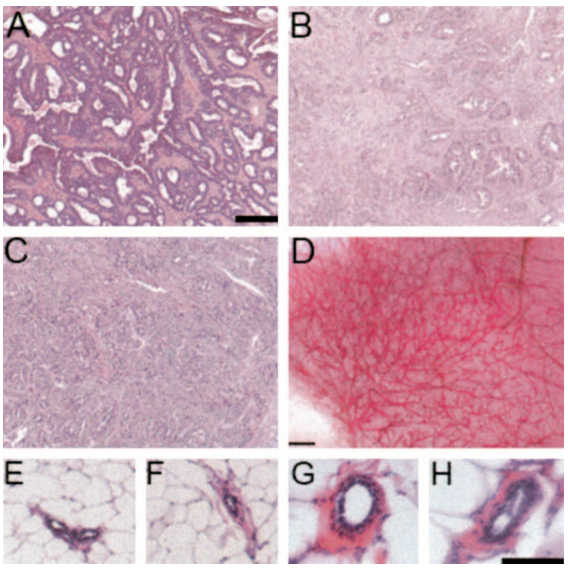

Figure 3. Diverse results were obtained from the same tumor donor. **A:** Papillary carcinoma used as a donor tumor. **B:** Secondary tumor developed in a 24-day-old host. **C:** Secondary tumor developed in a 52-day-old host. **D:** Normal ductal outgrowth developed in an 80-day-old host. In both secondary tumors there is a noticeable increase in the deposition of extracellular matrix and the number of glands is reduced, showing a less differentiated phenotype. **E** to **H** are a representation of the phenotypically normal ducts observed in the aged and parous hosts. Areas of the whole mounts containing ducts were removed, embedded in paraffin, sectioned, and stained with H&E. Scale bars: 50 μm (**G**, **H**); 100 μm (**A**, **B**, **C**, **E**, **F**); 2 mm (**D**).

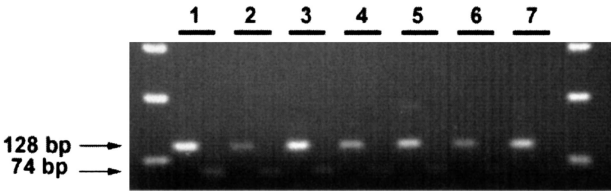

Figure 4. Examples of Ha-*ras*-1 expression in donor tumors and their outcomes. Each number represents one sample and its Ha-*ras*-1 expression: the **left lane** is the endogenous Ha-*ras*-1 and the **right lane** is the mutated gene. Samples 1, 3, and 5 are examples of donor tumors. Sample 2: DNA was extracted from a normal ductal outgrowth developed in an 80-day-old host injected with neoplastic cells from sample 1. Sample 4: DNA was extracted from a secondary tumor developed after the transplant of cells from sample 3. Samples 6 and 7: DNA was extracted from a normal ductal outgrowth and a secondary tumor developed in 80- and 24-day-old hosts, respectively. Both hosts were inoculated with sample 5. All donor tumors carry the codon 12 GGA to GAA mutation and the same mutation can be seen in both types of secondary outcomes, ie, tumors or normal ductal development.

We performed histopathological analyses of donor and secondary tumors as well as the microscopic neoplastic lesions following the criteria described by Russo and colleagues.[32] The donor tumors were carcinomas, papillary and cribriform types; the secondary tumors were classified mostly as infiltrating carcinomas, cribriform and comedo types. Figure 3 shows an example of a donor tumor and the outcome of the transplantation of its neoplastic cells into a 24-, 52-, and 80-day-old host. As mentioned above, tumors developed only in the younger animals.

*Mutated Ha-*ras*-1 Gene Expression Is Seen in Secondary Tumors and Ducts*

To recognize the tumor cells that were injected into the host's CFPs, we used the codon 12 GGA to GAA mutation in Ha-*ras*-1 gene as a marker of tumor origin. This marker was chosen because it has been claimed that *N*-nitrosomethylurea induces this particular point mutation in the Ha-*ras*-1 gene of mammary epithelial cells.[33] All of the donor tumors carried the codon 12 mutation and the same mutation was observed in both types of secondary outcomes, namely, tumors or normal ductal development, a confirmation of their tumor origin (Figure 4).

Discussion

The data collected suggest that an inoculum of just 5×10^4 neoplastic epithelial cells transplanted into the mammary stroma of syngeneic hosts resulted in tumor takes as well as normal ducts. This is consistent with the observations of Rivera and Vijayaraghaven[17] and Alston-Mills and Rivera.[28] Significantly, we also uncovered that the neoplastic outcome depended on the age of the host and/or their parity status at the time the epithelial cells were inoculated.

The development of the mammary gland is regulated by hormonal cues triggered by puberty and pregnancy. These cues orchestrate stromal-epithelial interactions leading to ductal growth, invasion, lateral branching, and alveolar development.[34] In our experiments, the time

points for donor tumor cell and stroma recombination were chosen to represent particular developmental stages of the normal mammary gland. *A priori*, we assumed that the CFP underwent developmental changes similar to those observed in the intact mammary gland. We based this assumption on the fact that both the stroma and the epithelium respond to ovarian hormones during the postnatal development of the mammary gland. Furthermore, some aspects of epithelial development are influenced by signals initiated in the stroma. For instance, Cunha and colleagues[35] observed that mammary ductal growth and branching during puberty are dependent upon estradiol signaling through the estrogen receptor-α present in the stroma cells.

We chose two time points during which ductal invasion of the stroma takes place in the intact gland, namely 24 days of age (the beginning of ductal invasion) and 52 days of age (when evident ductal growth is underway). This latter age also represents the well-known window of maximal vulnerability to chemical carcinogens in tumor-susceptible strains of rats.[36,37] The other time points were 80 days of age, when the ducts reach the edge of the fat pad, and 150 days of age, when the mammary gland of a virgin animal is considered an organ where no major tissue remodeling is observed.[38,39] We also took into account the fact that there is an inverse correlation between mammary tumor incidence and the age at which the carcinogen is administered.[30,36,40,41] We observed that the CFPs of younger animals (24 to 52 days of age) allowed for maximal secondary tumor development as well as ductal growth, whereas aged stroma (80 to 150 days of age) shifted the outcome toward normal ductal growth and a lower incidence of secondary tumors. In other words, we verified an inverse correlation between age and the detection of neoplasms that parallels the relationship between age and susceptibility to carcinogens in the mammary gland.

The mammary stroma undergoes biochemical and cellular changes associated with the endocrine milieu. The extracellular matrix components of rat mammary gland stroma are modified by the animal's reproductive state.[42] More recently, Schedin and colleagues[43] observed that the mammary matrix isolated from parous rats loses the ability to promote complex glandular development when compared to the matrix isolated from nulliparous mammary glands. Noncarcinogenic mouse mammary epithelial FSK-3 cells grown in a three-dimensional culture formed duct-like structures that invaded the substratum when cultured onto matrix from nulliparous 52-day-old rats. In contrast, the presence of matrix from parous rats restricted the formation of complex structures.[43] Herein, we observed that the stroma of parous rats not only restricted the development of a secondary tumor but, more importantly, instructed the neoplastic epithelial cells to form normal ductal outgrowths. Both Schedin and colleagues'[43] and our study strongly suggest that cellular and extracellular components of the stroma contribute to the protective effect of pregnancy against tumor formation. In addition, the stroma also plays a main role in the reversal of the neoplastic phenotype (Table 1, Figure 3). Moreover, the results presented herein suggest that the development of a protective effect against tumor formation observed in these animals does not require the contribution of the epithelial compartment, because the ductal epithelium was removed from the mammary gland at 10 days of age. It seems premature at this time to suggest which of the numerous cellular and extracellular stroma components play a definitive role in either the carcinogenic process or in its reversion.

Can these results in rodent mammary glands be extrapolated to clinical and epidemiological data in human breast cancer? The long-term outcome of survivors of the 1945 Hiroshima and Nagasaki nuclear explosions represents a relevant subject for comparison. The dose-specific excess relative risk for breast cancer increased 13-fold in women exposed before age 20 who went on to develop clinical cancer decades later,[44] whereas this risk was significantly lower in older women. This suggests that susceptibility to radiation decreases with age. Epidemiological data also show that the frequency of *in situ* breast carcinoma is higher in middle-aged women compared to the frequency of invasive carcinoma found in the elderly.[45,46] This pattern, in which the presence of ductal carcinoma *in situ* alone or associated with invasive carcinoma decreases with age, was reported in a more recent study by Wazer and colleagues.[47] It has been proposed that this lack of correlation between age and incidence is compatible with spontaneous regression of subclinical lesions.[26]

Finally, these experiments add to the mounting evidence that the stroma plays a crucial role in carcinogenesis and its reversion. The precise role of its diverse components deserves to be explored aggressively.

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